APPENDIX G
Laboratory Standard Operating Procedures (City of Los Angeles)

Chromogenic Substrate Method: Shoreline/Marine Samples

- **Procedure**
  - Disinfect the workbench area with 70% ethanol. Let air-dry.
  - Preparation of sample container
    - You will need one sterile container per sample. Label each sample container with station name and test to be performed (e.g., Container 1: S01, TC/EC, Container 2: S01, Entero).
    - Remove the outer plastic ring/label seal around the container cap. Remove the container cap, being careful not to touch the inside of the cap. Pour sterile deionized water from a flask into each container. Be careful not to touch the rim of the deionized flask or the container. Pour the D.I. water to the 100 mL mark on each container and replace the cap. Replace the cap back onto the D.I. water flask if there is any D.I. water left in the flask.
    - If a 10 mL sample aliquot is to be used, remove 10 ml of D.I. water from all sample containers using a sterile 10 ml pipet. If only 1 ml of sample is to be analyzed, skip this step of removing 10 ml of D.I. water.
    - You will need one Coli-18 reagent pak for each sample container labeled TC/EC and one Enterolert reagent pak for each container labeled Entero. Carefully separate one reagent snap pak from the strip, taking care not to accidentally open the adjacent pak. Tap the snap pak to ensure that all of the reagent powder is in the bottom part of the pack.
    - Open the pak by snapping back the top at the score line. Do not touch the opening of the pak.
    - Add the reagents to the appropriate sample containers filled with D.I. water. Replace the cap on the container, tighten, and gently mix until the reagent is dissolved. Note that when the Coli-18 reagent is added to the D.I. water in the container, the solution is a clear color and when Enterolert reagent is added to the D.I. water, the solution is a yellow color.
    - Pipet 10 mL of each sample into the appropriate sample containers. Place the used pipets into the pipet biohazard container. Replace the sample container caps and mix gently.

  - Quanti-tray/2000
    - Turn on Quanti-tray® sealer at the start of sample preparation.
    - You will need **one** Quanti-tray for **each** labeled sample container.
    - Check to see that the green Ready Light (above the amber power light) is illuminated on the sealer. The sealer will not operate until both the amber power light and the green Ready Light are

illuminated.

- Using one hand, hold a Quanti-tray upright with the well side (plastic) facing your palm. Squeeze the upper part of the Quanti-tray so that it bends towards the palm of your hand. Using your other hand, gently pull the foil tab at the top of the tray to separate the foil from the top of the tray, creating an open pouch. Avoid touching the inside of the foil or tray and be careful not to tear the foil.
- Pour the reagent/sample mixture directly into the Quanti-tray, avoiding contact with the foil tab at the top of the tray. Tap the small wells at the bottom of the tray to release any air bubbles. Allow any foam present to settle.
- Place the sample-filled tray onto the rubber insert of the sealer with the well side (plastic) of the tray facing down. Align the small and large wells with their corresponding holes in the rubber insert. Make sure the tray is properly seated in the rubber insert. With your hand, gently press on the back of the tray to distribute some of the liquid into the larger wells.
- Slide the rubber insert into the sealer until the motor grabs the rubber insert and begins to draw it into the sealer.
- In approximately 15 seconds, the tray will be sealed and partially ejected from the rear of the sealer. Remove the rubber insert and tray from the rear of the sealer.
- If a misaligned tray is accidentally fed into the sealer, press and hold the "**reverse"** button (located on the top, front center of the sealer). This will reverse the motor and you can then remove the tray. Do not reverse the motor once the rubber insert has been drawn fully into the input slot of the sealer.
- Repeat for each labeled tray. Turn off the sealer and unplug the unit when you are finished sealing all the trays.
- Using a felt-tipped marker, label the front of each tray with the incubation time.
- Place all Quanti-trays labeled "TC/EC" into the 35°C (Total coliform) incubator for 18 hours.
- Place all Quanti-trays labeled "Entero" into the 41°C (Enterococcus) incubator for 24 hours.

- QA Controls
  - Refer to QA/QC SOP
- Clean-up
  - Dispose of the empty, used sample container in the large, red biohazard containers.
  - Dispose of all pipet wrappers and empty reagent packs in the regular trash receptacle. Return all lab supplies to their proper storage areas.
  - Disinfect the workbench area with 70% ethanol. Let air-dry.
  - Discard original sample remaining in sample bottle (can discard down sink drain). Rinse with tap water and place empty bottles on

trash cart for later cleaning.

- Reading Quanti-Tray Sample Results
  - Disinfect the workbench area with 70% ethanol. Let air-dry.
  - TOTAL COLIFORMS - read 18 hours after incubation.
    - Remove the Quanti-trays from the 35°C (Total coliform) incubator.
    - Record the date, time, and analyst name or initials on the sample data sheet for the reading of Total Coliforms.
    - Compare the intensity of the yellow color of the sample wells to the intensity of the yellow color of the Comparator Quanti-tray. Any well with a yellow color of equal or greater intensity than the Comparator is considered a "positive" well. Wells with a clear color or a yellow intensity less than the Comparator are considered as "negative." **If reaction is unclear or borderline yellow, replace the tray in incubator for further incubation up to a total of 22 hours.**
    - Count the number of positive large wells. Remember that the single, large well at the very top of the Quanti-tray should also be included in the count if it is positive. Record the number of positive large wells on the sample data sheet. Count and record the number of large positive wells for each sample dilution that was set.
    - Count the number of positive small wells. Record the number of positive small wells on the sample data sheet. Count and record the number of small positive wells for each sample dilution that was set.

  - *E. COLI* - read 18-22 hours after incubation.
    - These results are read from the Total coliform Quanti-trays.
    - Record the date, time, and analyst name or initials on the sample data sheet for the reading of *E. coli*.
    - Place Quanti-tray under a UV cabinet or lamp.
    - Press the red button on the top of the UV lamp to turn the lamp on. Make sure the lamp is pointed away from you.
    - Count the number of large and small fluorescent wells for each sample dilution. Remember that the single, large well at the very top of the Quanti-tray should also be included in the count for the large wells if it is positive. Record the results on the sample data sheet.
    - If in doubt as to the fluorescence of a well, compare it to the negative fluorescence of the Quanti-tray Comparator. This Comparator is "negative" for fluorescence. **If fluorescence on the well(s) is/are still questionable, mark the well(s) with an indelible pen or marker and re-incubate Quanti-tray for an additional 2 - 4 hours**. Read Quanti-tray again following the incubation period.

ENTEROCOCCUS - read 24-28 hours after incubation

- Remove the Quanti-trays from the 41°C (Enterococcus) incubator.
- Record the date, time, and analyst name or initials on the sample data sheet for the reading of Enterococcus.
- Place Quanti-tray under a UV cabinet or lamp
- Press the red button on the top of the UV lamp to turn the lamp on. Make sure the lamp is pointed away from you.
- Shine the UV lamp directly on the sample Quanti-tray within five inches of the tray. Count the number of large and small fluorescent wells for each sample dilution. Remember that the single, large well at the very top of the Quanti-tray should also be included in the count for the large wells if it is positive. Record the results on the sample data sheet. Record the results on the sample data sheet.
- If in doubt as to the fluorescence of a well, compare it to the negative fluorescence of the Quanti-tray Comparator. This Comparator is "negative" for fluorescence. **If fluorescence on the well(s) is/are still questionable, mark the well(s) with an indelible pen or marker and re-incubate Quanti-tray for an additional 2 – 4 hours**. Read Quanti-tray again following the incubation period.

- When finished reading all the Quanti-trays, turn off UV lamp and dispose of all trays into the large red biohazard containers.
- Disinfect the workbench area with 70% ethanol. Let air dry.
- Leave the sample data sheets on the clipboard by the Quanti-tray sealer.

- Quanti-Tray Calculations
  - Enter the number of positive large and small wells into the Idexx generator or read from the Idexx MPN table. Multiply the number given in the table by the dilution factor used. If more than one dilution generates a result, take the average.

    Example # Positive large wells: 23

    # Positive small wells: 16

    Idexx MPN table: 52.7

    Calculation (10 ml aliquot of sample):

    52.7 (**number from table**) x 10 (**Result based on a 100 ml sample size**) = 530 MPN/100 ml

Membrane Filtration Method (for Enterococci analysis)

- **Media Preparation**
  - mEndo Agar LES
    - To rehydrate the medium, suspend 51 grams in 1 liter deionized

water containing 20 mL 95% ethanol and heat to boiling to dissolve completely. Cool to 45-50°C. (If using the agarmatic, follow the agarmatic directions for making mEndo.) Aseptically dispense 4-5 mL amounts into the lower halves of 60x15 mm sterile, disposable Petri dishes and allow to solidify. Final pH 7.2 ± 0.2. Record pH results in the media prep logbook.

- Set QA media controls.
  - Refer to QA/QC SOP
- Place agar plates in a labeled media container and refrigerate until needed. The holding time for agar plates is two weeks.

- mFC Agar
  - To rehydrate the medium, suspend 52 grams in 1 liter deionized water and heat to boiling to dissolve completely. Add 10 mL of a 1% solution of rosolic acid in 0.2 *N* NaOH. Continue heating for 1 minute. Cool to 45 -50°C. (If using the agarmatic, follow the agarmatic directions for making mFC.) Aseptically dispense 4-5 mL amounts into the lower halves of 50-60x15 mm tight-fitting sterile, disposable Petri dishes and allow to solidify. Final pH 7.4 ± 0.2. Record pH results in the media prep logbook.

  - 1% Rosolic Acid Solution - Add 0.1 grams rosolic acid to 10 mL of stock 0.2 *N* NaOH. Mix well.

  - Stock 0.2 *N* NaOH - Add 0.8 grams NaOH to 100 mL deionized water. Mix to dissolve. Store in a labeled polyethylene reagent bottle.

  - Set QA media controls.
    - Refer to QA/QC SOP
  - Place agar plates in a labeled Tupperware container and refrigerate until needed. The holding time for agar plates is two weeks.
- mE Agar
  - To rehydrate the medium, suspend 7.12 grams in 100 mL of deionized water. Heat to boiling to dissolve completely. Autoclave for 15 minutes at 121°C. Promptly remove from the autoclave and cool to 45-50°C. Add 0.024 grams Nalidixic Acid and 1.5 mL of a 1% solution of triphenyl tetrazolium chloride (TTC). (If using the agarmatic, follow the agarmatic directions for making mE.) Aseptically dispense 4-5 mL amounts into the lower halves of 60x15 mm sterile, disposable Petri dishes and allow to solidify. Final pH 7.1 ± 0.2. Record pH results in the media prep logbook.

    1% TTC Solution - Add 1 gram TTC to 100 mL of deionized water. Mix well. Using a sterile 0.22µm Millex-GS filter, filter-

sterilize the solution into a sterile, labeled 500 mL reagent bottle. Store in the refrigerator.

- Set QA media controls.
  - Refer to QA/QC SOP
- Place agar plates in a labeled Tupperware container and refrigerate until needed. The holding time for agar plates is two weeks.

- Esculin Iron Agar (EIA)
  - To rehydrate the medium, suspend 1.65 grams in 100 mL of deionized water. Heat to boiling to dissolve completely. Autoclave for 15 minutes at 121°C. Promptly remove from the autoclave and cool to 45-50°C. (If using the agarmatic, follow the agarmatic directions for making EIA.) Aseptically dispense 4-5 mL amounts into the lower halves of 60x15 mm sterile, disposable Petri dishes and allow to solidify. Final pH 7.1 ± 0.2. Record pH results in the media prep logbook.
  - Set QA media controls.
    - Refer to QA/QC SOP
  - Place agar plates in a labeled Tupperware container and refrigerate until needed. The holding time for agar plates is two weeks.
- Phosphate-Buffered Water
  - 1 *N* NaOH - Carefully add 4 grams NaOH to 100 mL deionized water. Mix to dissolve. Store in a labeled polyethylene reagent bottle.
  - Stock Phosphate Buffer Solution - add 34.0 grams potassium dihydrogen phosphate (KH_2PO_4) to 500 mL deionized water and mix to dissolve. Adjust pH to 7.2 ± 0.5 with 1 *N* NaOH and bring volume to 1 liter, using a 1-liter volumetric flask. Transfer to a reagent bottle and autoclave for 15 minutes at 121°C. Let cool and refrigerate. Discard if turbidity is present.
  - Stock Magnesium Chloride Solution - add 81.1 grams $MgCl_2 \cdot 6H_2O$ to 1 liter deionized water and mix to dissolve. Transfer to a reagent bottle and autoclave for 15 minutes at 121°C. Let cool and refrigerate. Discard if turbidity is present.
  - Working Solution of Phosphate-Buffered Dilution/Rinse Water
    - Add 1.25 mL stock phosphate buffer solution and 5 mL stock magnesium chloride solution to 1 liter deionized water. Adjust pH to approximately 7.6-7.7 with 1 *N* NaOH. Mix and dispense approximately 9.5 mL into specially marked dilution test tubes. Autoclave at 121°C for 15 minutes. If phosphate-buffered rinse water is needed, autoclave 1-2 L volumes in large flasks for 45 minutes at 121°C.
    - Cool and check that buffered water level is at the marked line (9 mL) on the test tube. Aseptically adjust water level if necessary. Tightened test tube or flask caps and store at

room temperature. Holding time for screw-capped media is 3 months. Final pH 7.2 ± 0.1.

- Sterility control - test the sterility of the buffered dilution water by aseptically pouring 2 test tubes of dilution water into a sterile bottle containing 100 mL of Tryptic Soy Broth. Test the sterility of the liter flasks of rinse water by aseptically adding 20 mL buffer to a sterile bottle containing 100 mL TSB. Incubate the bottle for 48 hours at 35.0 ± 0.5°C. Record pH and sterility check results in the media prep logbook.

- Tryptic Soy Broth (TSB)
  - To rehydrate the medium, suspend 30 grams in 1 liter of deionized water and mix to dissolve completely. Dispense 100 mL of broth into 125 mL Nalgene bottles. Autoclave for 15 minutes at 121°C. Promptly remove from the autoclave when done. Let cool and then tighten caps. Final pH 7.3 ± 0.2. Record pH results in the media prep logbook.
  - Set QA media controls.
    - Refer to QA/QC SOP
  - Place TSB bottles in the refrigerator until needed. The holding time for screw-capped media is three months.

- **Plate Labeling Procedure**
  - Clean and wipe the bench-top work area with 70% ethanol and let air dry.
  - Check the monthly sample calendar for the samples and duplicates scheduled for the day.
  - Check the QA results of the prepared agar plates to be used. These results are recorded in the media prep logbook. Use only media that have passed the sterility, positive control, and negative control checks.
  - Record the media preparation dates for all the agar plates being labeled. The dates are recorded in the media prep logbook under "Prep Date of Media in Use."
  - Inspect all agar plates.
    - Discard any plates that have bubbles that will interfere with bacterial growth when the membrane filter is placed on the agar surface.
    - Check plates for contamination of any kind (bacterial growth, mold, or strange color). Discard any contaminated plates into a biohazard bag.
  - Using an indelible marking pen or pre-printed labels, label each plate with the station name or location at the top of the Petri dish, sample volume or dilution in the middle, and sample date at the bottom of the dish.
    - Consult the Sample Dilution Table for the necessary dilutions for each sample type.
    - mEndo and mFC agar plates are labeled on the bottom (agar side) of the Petri dish.

        - mE agar plates are labeled on the top (lid side) and the bottom (agar side) of the Petri dish.
    - Stack all the agar plates for the same station together after the plates are labeled. Stack plates by ascending volume order (smallest volume on top).
    - When stacking, be sure to place all plates, agar side up.
    - Place the stack of plates for each sample into a slot in one of the agar plate carriers.
    - Add a small stack of unlabelled mEndo agar plates to the carrier. These plates will be used for QA blanks as needed during filtering.
    - Label the cover of each plate carrier with the sample stations or locations for all plates in the carrier. Include duplicate stations on the label for all boat plate carriers.
    - If plates are labeled one day in advance of use, refrigerate the plate carriers. Labeled plates that are refrigerated need to be taken out of the refrigerator on the day of use.
    - If plates are labeled on the day of use, the plate carriers can be left out at room temperature until needed.

- **Filtration Procedure**
    - Clean and wipe the bench top work area with 70% ethanol and let air dry.
    - Gather the necessary filtration equipment.
    - Aseptically transfer sterile, phosphate-buffered rinse water into a sterile squirt bottle.
    - Select samples to be filtered. Select the proper agar plates for the samples and check the plate stacking order to make sure sample volumes are in ascending order.
    - Make 1:10 serial dilutions (if needed).
        - Shake the sample vigorously for several seconds (about 25 - 30 times) to break up any bacterial cell aggregates, to separate cells from particulate matter, and to make the sample homogenous.
        - Aseptically pipet 1 mL of the sample into a sterile 9 mL dilution test tube and shake or vortex vigorously. This is a 1:10 (10^{-1}) dilution of the sample.
        - Aseptically pipet 1 mL of the 10^{-1} dilution into a second 9 mL dilution tube and shake or vortex vigorously. This is a 1:100 (10^{-2}) dilution.
        - Aseptically pipet 1 mL from the second (10^{-2}) dilution tube into a third 9 mL dilution tube and shake or vortex vigorously. This is a 1:1000 (10^{-3}) dilution.
        - Continue making 1:10 serial dilutions as needed.
    - Fill the alcohol lamp with 95% ethanol and light it.
    - Prepare filtration equipment, one filtration unit per sample.
        - Wipe the Microfil support base with an alcohol pad. Let dry.
        - Remove filter screen disc from the 95% alcohol jar using the long-handled forceps. Gently shake the disc over the alcohol jar to

remove any excess alcohol. Flame-sterilize the disc. Allow flame to self-extinguish. Place disc onto the Microfil support base.
    - Squirt the disc with a small amount of sterile buffer to wash any residual alcohol off the disc. Apply vacuum to drain the buffer off the disc.
    - Aseptically remove a membrane filter from the filter dispenser, using an alcohol flame-sterilized forceps. Place the filter, grid-side-up on filter support base.
    - Aseptically remove a sterile, disposable Microfil funnel from the funnel dispenser.
    - Put the funnel over the filter on the support base. Place thumbs and index fingers of both hands on the upper, outside ridge of the funnel. Evenly push down on the funnel to securely lock it into place.
- Shake sample vigorously for several seconds (about 25 - 30 times) to break up any bacterial cell aggregates, to separate cells from particulate matter, and to make the sample homogenous. Place bottle at a slant to let any sand or debris in the sample settle to the bottom sides of the bottle.
- Record filtering start time and initials in the LIMS "Micro Log-in" Excel worksheet on the PC computer. Move the cursor to the appropriate cell for the sample being filtered.
    - If the starting time is the current time, press "CTRL+T."
    - Alternately, enter the time using a colon, ex. "10:25 or 14:00."
- Before filtering the sample, determine if a QA sterility blank needs to be done.
    - Refer to QA/QC SOP
- Wet the membrane filter with an adequate amount of sterile rinse water before adding sample aliquots delivered with a pipet. Add the sample aliquot to the filter according to the plate stacking order. Use a new filter for each sample aliquot.
- Use sterile pipets for sample volumes < 20 mL. If the pipet is to be used again, rest the pipet tip against the inner lip of the sample bottle. Do not let the pipet tip rest on the bottom of the sample bottle. Discard used pipets into the pipet biohazard container.
- For sample volumes of 50 mL or 100 mL, aseptically pour the sample to the measured lines on the Microfil funnel. If an excess amount of sample is poured into the funnel, use a sterile pipet to remove the excess. Discard the excess sample along with the pipet into the pipet biohazard container.
- Before applying the vacuum, swirl the sample in Microfil funnel by moving the funnel in a gentle circular motion to evenly distribute bacterial cells on the membrane filter surface.
- Apply vacuum, letting the sample drain through the filter.
- Thoroughly rinse down the walls of the funnel two times with a generous amount of sterile buffer water. This will wash down any bacteria that may adhere to the sides of the funnel.
- With one hand on the outside walls of the funnel, use a backwards and

upwards motion to pop the funnel off the support base. Continue to hold the funnel with your hand. Use your other hand to remove aseptically the filter with a flame-sterilized forceps (one sterile forceps per membrane filter). Aseptically replace the funnel back on the support base.

- Aseptically place the filter on the surface of the appropriate agar plate, using a rolling motion to avoid trapping air between the agar and the filter that will result in the formation of bubbles. If any air is trapped under the filter, reset the membrane filter onto the agar surface. Place the used forceps into the jar of ethanol.
- Stack finished plates by sample and media type. Remember to always position finished plates agar (bottom) side up. This is to avoid any condensation dripping onto the surface of the filter during incubation, which may interfere with or distort bacterial growth.
- Continue filtering the sample, following the steps detailed above for each sample volume or dilution labeled on the stack of plates.
- If a duplicate sample is being filtered, the same pipets and dilution tubes (if needed) may be used for both the regular sample and the duplicate sample.
- When the sample is finished being filtered, place mEndo and mE agar plates in a covered incubation container (with moist sponges) according to media type. Total coliform mEndo agar plates are incubated for 24 ± 2 hours at 35.0 ± 0.5 °C. Fecal coliform mFC agar plates are incubated for 24 ± 2 hours at 44.5.0 ± 0.2 °C. It is important that these plates be incubated within 20 minutes of filtration to ensure heat-shock of the non-fecal bacteria. Plates are incubated in either the dry heat-sink incubators or sealed in waterproof bags and placed in the 44.5 ± 0.2°C water bath. Enterococcus mE agar plates are incubated for 48 ± 2 hours at 41.0 ± 0.5°C.
- Record filtration finish time, initials, and incubation time in the LIMS "Micro Log-in" Excel worksheet on the PC computer.
- The incubation containers should be labeled with the indicator bacteria, test date, and incubation time.
- Place used Microfil funnels in the biohazard bag for the funnels. Place sample bottles, empty buffer flasks, and used squirt bottles (if not being used for filtering more samples) in a tub for later washing.
- Wipe down the bench-top work area with 70% ethanol and let air dry.
- To filter another set of samples, wipe the Microfil support base and filter screen disc with a new alcohol pad. Rinse the disc with sterile rinse water. Repeat procedure as detailed in the above sections.
- When taking a long break between filtering samples, wipe the Microfil support base and filter screen disc with a new alcohol pad. Leave the alcohol pad on the screen disc. Place an alcohol-wiped cap over the Microfil unit. Before filtering again, remove the cap and re-wipe the Microfil unit and filter screen disc with the alcohol pad. Rinse the disc with sterile rinse water. Repeat procedure as detailed in the above sections.

- When all samples have been filtered, remove the filter screen disc from the Microfil support base and put in the 95% alcohol jar. Wipe the Microfil support base with a new alcohol pad. Leave the alcohol pad in the empty disc space. Place an alcohol-wiped cap over the Microfil unit.

- **Colony Counting Procedure**
  - Check the LIMS "Micro Log-in" Excel worksheet for the incubation times of the plates that need to be read that day. Determine when the plates can be read according to their required incubation times.
  - Gather the necessary data worksheets for all samples to be read. Each test and sample type has separate data worksheets.
  - Record the time the plates are read and analyst initials in the LIMS "Micro Log-in" Excel worksheet and on the data worksheets.
    - If the read time is the current time, press "CTRL+T."
    - Alternately, enter the time using a colon, ex. "10:25 or 14:00."
  - If desired, wear disposable gloves when handling and reading the plates.
  - Remove plates from the incubator when it is time to read them and arrange them in ascending volume order for each station.
  - Use the stereoscopic microscope with a fluorescent lamp to aid in identifying and counting colonies.
  - Starting with the control blank plate if one was done, examine the filter for bacterial contamination or any notable changes on the filter or agar media.
  - Examine and count all the plates set for a single sample, starting with the smallest sample volume filtered or the most dilute sample.
  - Colonies that have grown into each other should be counted individually. Separate nuclei or a fine line of contact may usually be seen.
  - Colonies in every filter grid square within the filtering area are to be counted.
  - To make counting easy and simple, start counting at the top of the filter. Count from left to right, following the grid lines, and continue to the bottom of the filter.
  - Countable ranges - Due to the possible adverse effect of colony crowding on sheen or color development on the filter membrane, and to be assured of a statistically valid colony count, minimum and maximum bacterial levels have been set for each of the indicator bacteria.
    - Total bacteria: <200 total colonies (background and indicator bacteria).
    - Total Coliform: 20 - 80 coliform colonies
    - Fecal Coliform: 20 - 60 fecal coliform colonies
    - Enterococcus: 20 - 60 Enterococcus colonies
  - Colony Morphology
    - Total Coliforms
      - The typical colony has a pink to dark-red color with a shiny, greenish-gold, metallic surface sheen. The sheen may cover the entire colony, or it may appear only in the central area or on the periphery.

        - This sheen is produced as a by-product of lactose fermentation (acid aldehyde complex) in combination with the Schiff's reagent (fuschin sulfite) in the mEndo media.
    - Fecal Coliforms
        - Any colony exhibiting any light or dark blue color, whether covering the entire colony or only in or on part of the colony.
        - This blue color is a result of the acid produced by the fermentation of lactose combining with the aniline blue dye in the mFC media.
        - Colonies exhibiting a cream or grey color are not fecal coliforms.
    - Enterococcus
        - After 48 ± 2 hours incubation, mE filters with growth on them are transferred to room temperature EIA plates.
        - Using forceps, remove the filter (handling the filter by its edge, outside of the filtration area) from the mE plate and roll it onto the agar surface of the EIA plate.
        - Replace the top of the EIA plate with the labeled top lid of the original mE plate.
        - Incubate the EIA plates for 20 minutes at 41.0 ± 0.5°C.
        - Enterococci are pink to carmine-red colonies with black or reddish-brown precipitate or halos on the underside of the filter when placed on EIA agar.
        - The colony color is due to the reduction of the vital indicator TTC (2,3,5-Triphenyl tetrazolium chloride) to non-reversible formazin. The dark precipitate or halo is the result of the hydrolysis of esculin.
- Record all colony counts and any other notable information on the data worksheet. Comments should include information about unusual conditions on the filter, such as the presence of solids, artifacts, or high background counts. The condition of the growth on the filter should also be noted, such as confluent areas or confluent growth over the filter.
    - CG = confluent bacterial growth with indistinct or non-discrete colonies.
    - TNTC = Too Numerous To Count
    - >200 = greater than 200 background and indicator colonies on a filter.
- If there are any questions regarding counting colonies or any unusual or suspicious plates, save all plates for that sample and show them to a microbiologist.
- Dispose of all plates and gloves in a biohazard container. Autoclave at the end of the day.

- **Calculations**
  - Due to the possible adverse effect of colony crowding or color development on the filter membrane, and to be assured of a statistically valid colony count, minimum and maximum bacterial levels have been set for each of the indicator bacteria.
    - Total bacteria: <200 total colonies (background and indicator bacteria).
    - Total Coliform: 20 - 80 coliform colonies
    - Fecal Coliform: 20 - 60 fecal coliform colonies
    - Enterococcus: 20 - 60 Enterococcus colonies
  - Indicator bacteria are expressed as bacterial density (CFU) per 100 mL of sample.
  - The raw bacterial counts from the data worksheets are entered into LIMS "Sample Data Entry" Excel worksheets on the PC computer by a technician. The computer calculates the final bacterial densities for each sample and prints a copy of the data worksheet. See the LIMS Data Entry SOP for more details.
  - The supervisor verifies the daily-calculated bacterial densities. Daily bacterial density reports are printed out by the computer and E-mailed to the primary leads of the jurisdictional groups, who in turn will communicate this data to its jurisdictional members. The data reports are kept in a labeled notebook and the original data worksheets are kept in the data file cabinet. See the LIMS Data Validation SOP for more details.
  - If the final bacterial densities need to be calculated by hand, the following guidelines should be used. All calculated values should have only 1 or 2 significant figures, depending on the colony counts.

    - Countable Range (Standard Methods., EPA):

      Countable range number of colonies x 100 = (value) CFU/100 **mL filter volume**

    Disregard non-countable range counts and volumes.

Volume	Count	
blank	0	
0.5	0	
5.0	6	$\frac{35}{20} \times 100 =$ **180 CFU/100 mL**
20	35	
50	95	

    - Two volumes in the countable range (EPA):

      Calculate each count independently as in 6.4.1. above and then average the results.

Volume	Count		
blank	0	20 x 100 = 100	60 x 100 = 120
0.5	0	20	50
5.0	6		
20	20	100 + 120 = **110 CFU/100 mL**	
50	60	2	

- Counts less than the countable range (Standard Methods):

  Add all colonies x 100 = (value) CFU/100 mL
  Total all volumes

Volume	Count		
blank	0		
0.5	0		
5.0	1	19 + 4 + 1 + 0	x 100 = **32 CFU/100 mL**
20	4	50 + 20 + 5 + 0.5	
50	19		

- No counts on any filter volume (EPA):

  1 x 100 = < (value) CFU/100 mL
  Largest volume filtered

Volume	Count	
blank	0	
0.5	0	1 x 100 = **<2 CFU/100 mL**
5.0	0	50
20	0	
50	0	

- Counts greater than the countable range - too numerous to count (TNTC) or confluent growth (CG) (EPA):

Highest upper limit count x 100 = >(value) CFU/ 100 mL
Smallest volume filtered

Volume	Count	For Total Coliforms:
blank	0	80 x 100 = **>16,000 CFU/100 mL**
0.5*	TNTC or CG	0.5
5.0	TNTC or CG	For Fecal Coliforms or Enterococci:
20	TNTC or CG	60 x 100 = **>12,000 CFU/100 mL**
50	TNTC or CG	0.5

*NOTE: If the count at the lowest dilution is TNTC, try to estimate the

count on the plate. Estimate the count in a quadrant if necessary. Use this number to calculate the count per 100 mL.

- Confluent Growth Counts (Standard Methods, EPA):

  Disregard all dilution volumes that are confluent growth. Analyze remaining counts and volumes.

Volume	Count
blank	0
0.5	0
5.0	CG
20	CG
50	CG

$\frac{1}{0.5} \times 100 =$ **<200 CFU/100 mL**

Volume	Count
blank	0
0.5	3
5.0	20
20	CG
50	CG

$\frac{20}{5.0} \times 100 =$ **400 CFU/100 mL**

- Total bacterial count (background bacteria plus indicator bacteria) greater than 200 colonies (Std. Methods.):

  Analyze counts and volumes. Report as a greater than value.

Volume	Count
blank	0
0.5	0 (>200)
5.0	0 (>200)
20	CG
50	CG

$\frac{1}{5} \times 100 =$ **>20 CFU/100 mL**

Volume	Count
blank	0
0.5	0
5.0	3
20	18 (>200)
50	60 (>200)

$\frac{60}{50} \times 100 =$ **>120 CFU/100 mL**

- Total colonies less than 200, but indicator bacteria greater than upper limit (Std. Methods.):

  If plate has well isolated, discrete colonies that can be easily counted, use the higher count.

Volume	Count
blank	0
0.5	85
5.0	TNTC
20	TNTC
50	TNTC

Volume	Count
blank	0
0.5	2
5.0	95
20	TNTC
50	CG

$$\frac{85}{0.5} \times 100 = \textbf{17,000 CFU/100 mL} \qquad \frac{95}{5} \times 100 = \textbf{1,900 CFU/100 mL}$$